

NF- κ B activity in transgenic mice: developmental regulation and tissue specificity

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SUMMARY

The transcription factor family NF- κ B/Rel is responsible for the regulation of a large number of cellular genes and some viruses. Since there is a strong similarity between the NF- κ B/Rel family members and the *Drosophila melanogaster* protein DORSAL, which is activated early during embryogenesis, we were interested in determining the pattern of NF- κ B activity during mouse development. Two *lacZ* reporter constructs, each driven by promoter elements that are dependent on the presence of nuclear NF- κ B/Rel activity, were used to produce transgenic mice. The analysis of these mice did not identify nuclear NF- κ B/Rel activity in early development prior to implantation or during the gastrulation processes. Earliest expression of the *lacZ* transgene was detected on day E12.5. Before birth *lacZ* expression was seen in discrete regions of the rhombencephalon of the developing brain, in the spinal

medulla, in some of the blood vessels and in the thymus. After birth, the NF- κ B/Rel activity in the thymus remained but nuclear activity was also found in the bone marrow, in the spleen and in the capsule of the lymph nodes. In the central nervous system, drastic changes in NF- κ B/Rel activity could be observed in the first 3 weeks after birth, when the cortex and the cerebellum reach functional and morphological maturity. Considering the results of the p50, p65, relB and c-rel knock-out mice and our present findings, we believe that the NF- κ B/Rel proteins known so far are probably not implicated in processes of early development and differentiation of the different tissues, but rather in maintaining their function once matured.

Key words: transcription factor NF- κ B/Rel, transgenics, central nervous system, tissue specificity, mouse

INTRODUCTION

Nuclear factor κ B (NF- κ B) is a transcription factor implicated in the regulation of numerous cellular genes such as those encoding many cytokines, adhesion molecules, immunoreceptors, transcription factors, as well as some viruses including HIV (reviewed in Blank et al., 1992; Grilli et al., 1993; Baeuerle and Henkel, 1994; Siebenlist et al., 1994 and references therein). The active form of NF- κ B, which is capable of binding DNA, is a dimeric complex composed of heterodimeric or homodimeric combinations between several proteins belonging to the same family. The family is characterized by the presence of a ~300 amino acids region called the rel homology domain (RHD), which contains a dimerization and a DNA-binding domain, as well as a nuclear localization sequence. A transactivation domain is found in the C-terminal region of the NF- κ B subunit p65, relB, the product of the *c-rel* proto-oncogene and the *Drosophila melanogaster* homologues dorsal and dif. The most common species of NF- κ B are heterodimers of the p50 subunit and either p65 or the product of *c-rel*.

In mature B-cell, monocytic and some T-cell lines, certain NF- κ B/Rel complexes are constitutively present in an active form in the nucleus. However, other cells contain NF- κ B/Rel in an inactive form located in the cytoplasm where it is bound to inhibitors of the NF- κ B/Rel family called I κ B. In the rest of this paper, we will refer to these two types of activities as constitutive and inducible, respectively. The inhibitor proteins share a partially conserved domain of five to seven ankyrin motifs, responsible for the physical interaction with the NF- κ B/rel proteins (Blank et al., 1992; Nolan and Baltimore, 1992; Beg and Baldwin, 1993). Two members of the NF- κ B/Rel family, namely p50 and p52, are produced as the inactive precursors p105 and p100 respectively, which are also located in the cytoplasm and behave like I κ B molecules (Rice et al., 1992; Mercurio et al., 1993). Upon cell activation by cytokines, mitogens, phorbol esters and some viruses, the inhibitors are modified through site-specific phosphorylations, which target them for subsequent proteolytic degradation. This event releases the NF- κ B molecules, which can now be translocated into the nucleus and activate transcription of their target genes.

While so far no mammalian NF- κ B/Rel member has been

shown to play a role in developmental processes, DORSAL, one of the NF- κ B homologues in *Drosophila melanogaster* (Steward, 1987), is an essential morphogen responsible for the ventralization effect in developing *Drosophila* embryos (for a review see St. Johnston and Nüsslein-Volhard, 1992). During early developmental stages, DORSAL is kept in the cytoplasm by CACTUS, which is the homologue of the mammalian I κ B proteins. After about 10 nuclear division cycles, in response to a signal received by the cell surface receptor TOLL in the ventral part of the embryo, DORSAL enters the nucleus and activates expression of target genes.

Concerning mammalian NF- κ B/Rel members, earliest expression has been described by Carrasco et al. (1994) for *c-rel* in the thymus and in mesoderm-derived hematopoietic cells of the fetal liver at day E13.5, as well as for *relB* in the thymus at day E14 (Carrasco et al., 1993). This coincides with the appearance of Ia-bearing dendritic cells, which express *relB* (Carrasco et al., 1993). Nevertheless generally little is known about the actual patterns of NF- κ B/Rel activity during mammalian embryogenesis.

We therefore decided to analyze the NF- κ B/Rel activity pattern during mouse development. Since the NF- κ B/Rel proteins are expressed almost ubiquitously, the pattern of expression of transcriptionally active nuclear complexes cannot be determined by using immunohistochemistry or in situ hybridization. We thus chose to construct transgenic mice carrying a κ B-dependent reporter gene so that the identification of positive cells or tissues would reflect the distribution of nuclear NF- κ B/Rel activity. Two promoter constructs, whose activity is dependent on the presence of active nuclear NF- κ B/Rel complexes, were cloned upstream of the β -galactosidase (*lacZ*) reporter gene containing a nuclear localization sequence. The resulting transgenic mice showed that NF- κ B/Rel activity was not detected until late in development. These data support the findings of Carrasco et al. (see above; 1993, 1994), and of the NF- κ B/Rel knock-out experiments. With the exception of the p65 knock-out (Beg et al., 1995), which was lethal from day E16 on, due to apoptosis of the hepatocytes, inactivation of the *c-rel* (Köntgen et al., 1995), the *p50* (Sha et al., 1995) or the *relB* gene (Weih et al., 1995) were not embryonically lethal. Therefore our results confirm these data and suggest that known mammalian NF- κ B/Rel proteins are not involved in early developmental processes. In addition, we demonstrate here that a constitutive NF- κ B/Rel activity can be detected in specific regions of the brain and is the first activity to be detected during development.

MATERIALS AND METHODS

DNA constructs

A 3.1 kbp *NcoI-PstI* fragment derived from the p105 promoter and including three functional κ B sites (Ten et al., 1992; Cogswell et al., 1993), was isolated and cloned into pSKT, which has been linearized previously with *NcoI-PstI*. The construct was named p105lacZ. pSKT, kindly provided by S. Tajbakhsh, is derived from pBlueScript II SK⁺ and contains a nuclear localization sequence (NLS) upstream of the *E. coli* β -galactosidase gene and the SV40 polyadenylation site.

The negative control for p105lacZ, p105mutlacZ, consisted of the same promoter sequence, with the three κ B sites mutated. Mutagenesis of the p105 promoter was performed by the oligo-directed procedure, using the Bio-Rad kit (Hercules, USA). Three functional sites for the binding of NF- κ B/Rel complexes were previously

reported in the p105 promoter (Ten et al., 1992; Cogswell et al., 1993): GGGGGCTTCCC, found at position -71 (NF- κ B site 1; numbering is according to Ten et al., 1992), GGGCTTCCCC found at position +118 (NF- κ B site 3) and TGGGAATTTC found at position +212 (NF- κ B site 4). NF- κ B site 2 (-36) was shown to be non-functional. The NF- κ B sites 1, 3 and 4 were mutated into GCTC-CCTTCCC, CTCCTTCCCC and TCTCCATTTC, respectively, which no longer bind NF- κ B/Rel complexes. The mutant plasmid was verified by sequencing. Subsequently, it was cut by *XhoI* and *NcoI* and cloned into pSKT, as described above.

A third construct, (Ig κ)₃conalacZ, was produced using three copies of the NF- κ B/Rel-binding site of the immunoglobulin κ light chain enhancer, inserted immediately upstream of the conalbumin (cona) minimal promoter (Ten et al., 1992). This enhancer-promoter element was cloned into *Sall-HindIII* digested pSKT.

As negative control for this construct, we used the conalbumin promoter alone, cloned into *Sall-HindIII* of pSKT. The construct was denominated conalacZ.

Transfection experiments

The constructs mentioned above were tested by transfecting them into 293T cells using the CaPO₄ coprecipitation method. The β -galactosidase activity was quantified using the Tropic kit (Bedford, USA) and a luminometer (Berthold, Germany).

Production of transgenic mice

Transgenic mice were generated by injection of purified linearized DNA fragments into the pronucleus of C57Bl/6 \times SJL F1 zygotes (Hogan et al., 1994). All constructs mentioned above were digested with *XhoI* and *NoI* to isolate the promoter-enhancer-nls-*lacZ* fragment away from the vector sequences.

Microinjection was kindly performed by P. Marchand (Pasteur Institute Transgenic Facility). C57Bl/6 \times SJL females were used as recipients for the injected embryos. The same breed was also used for all further breedings and subsequent studies.

Genotyping of transgenic mice

Transgenic mice, carrying the constructs mentioned above were identified by PCR and Southern Blot analysis. Genomic DNA from tail biopsies was prepared according to Laird et al. (1991). PCR analysis was carried out according to Hanley and Merlie (1991).

In order to determine the copy number of the transgene, and whether the animals contained the full-length transgene, Southern Blot analysis was performed (Church and Gilbert, 1984) with 10 μ g of genomic DNA digested with either *NcoI*, cutting inside the transgene, or with *PstI/Sall* and *XbaI* to isolate the whole transgene.

Whole-mount β -galactosidase staining

Pre- and postimplantation embryos as well as single organs were fixed for 20-30 minutes at 4°C in 1% formaldehyde and 0.2% glutaraldehyde in phosphate-buffered saline (PBS). X-Gal staining was performed at 30°C in 4 mM potassium ferrocyanide, 4 mM potassium ferricyanide, 2 mM MgCl₂, and 400 μ g/ml X-gal (4-chloro-5-bromo-3-indolyl- β -galactoside, Sigma) in PBS for 16 hours. Afterwards, embryos and organs were washed several times in PBS and postfixed in the fixing solution mentioned above. Pictures of organs and embryos were taken using a Leica binocular microscope (Wild M10), attached to a video camera system (Sony).

Histological analysis of whole-mount embryos and brains

For histological analysis, the embryos and organs were frozen and embedded in Tissue-Tek OCT compound (Miles-Bayer). 20 μ m sections were cut using a cryostat and collected on gelatine-coated slides. These sections were fixed, X-gal stained as described above and counterstained with neutral red. Sections were dehydrated and mounted using EUKITT, photographed on a Nikon microscope equipped with a camera, using Kodak Ektachrome 64T film.

In order to differentiate between neurons and glia, double immunofluorescence staining were performed with anti-GFAP (glial fibrillary acidic protein) antiserum (DAKO) or anti-neurofilaments NF160 monoclonal antibody (Sigma) and anti- β -galactosidase antibodies (CAPPEL and SIGMA). Secondary antibodies coupled to fluorescein

(SANOFI DIAGNOSTIC PASTEUR) or to Texas-Red (VECTOR) were used to reveal the primary antibodies. Optical sections were obtained with a confocal laser scanning microscope (Zeiss) and recorded on Ektachrome films (KODAK).

Histological analysis of the lymphoid organs

Thymus, spleen and the latero-aortic lymph nodes from 3-month-old mice were dissected and immediately fixed in PBS with 1% formaldehyde and 0.2% glutaraldehyde for 20 minutes at 4°C. After removing

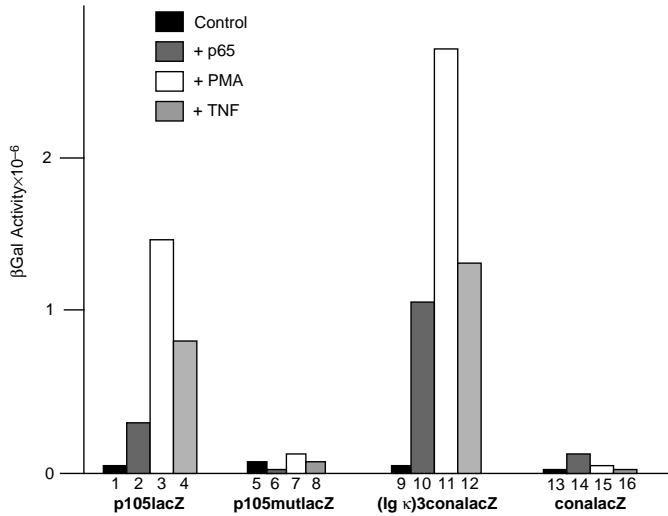


Fig. 1. Transcriptional activity of κ B-dependent *lacZ* constructs. 293T cells were transfected by the calcium phosphate coprecipitation method with the following constructs: lanes 1 to 4, p105lacZ, containing the promoter of the p105 protein including three NF- κ B/Rel-binding sites; lanes 5 to 8, p105mutlacZ, identical to the p105lacZ construct except for the 3 κ B sites which have been mutated; lanes 9 to 12, (Ig κ)₃conalacZ, containing three copies of the κ B site from the immunoglobulin κ light chain gene enhancer in front of the conalbumin minimal promoter; lanes 13 to 16, conalacZ, containing only the conalbumin minimal promoter. The transfected cells were either co-transfected with the CMV-p65 expression vector (lanes 2, 6, 10 and 14) or stimulated by PMA (lanes 3, 7, 11 and 15) or TNF (lanes 4, 8, 12 and 16) for 18 hours. The activity of the reporter gene was quantified 48 h after transfection. β -gal activity is expressed as RLU units.

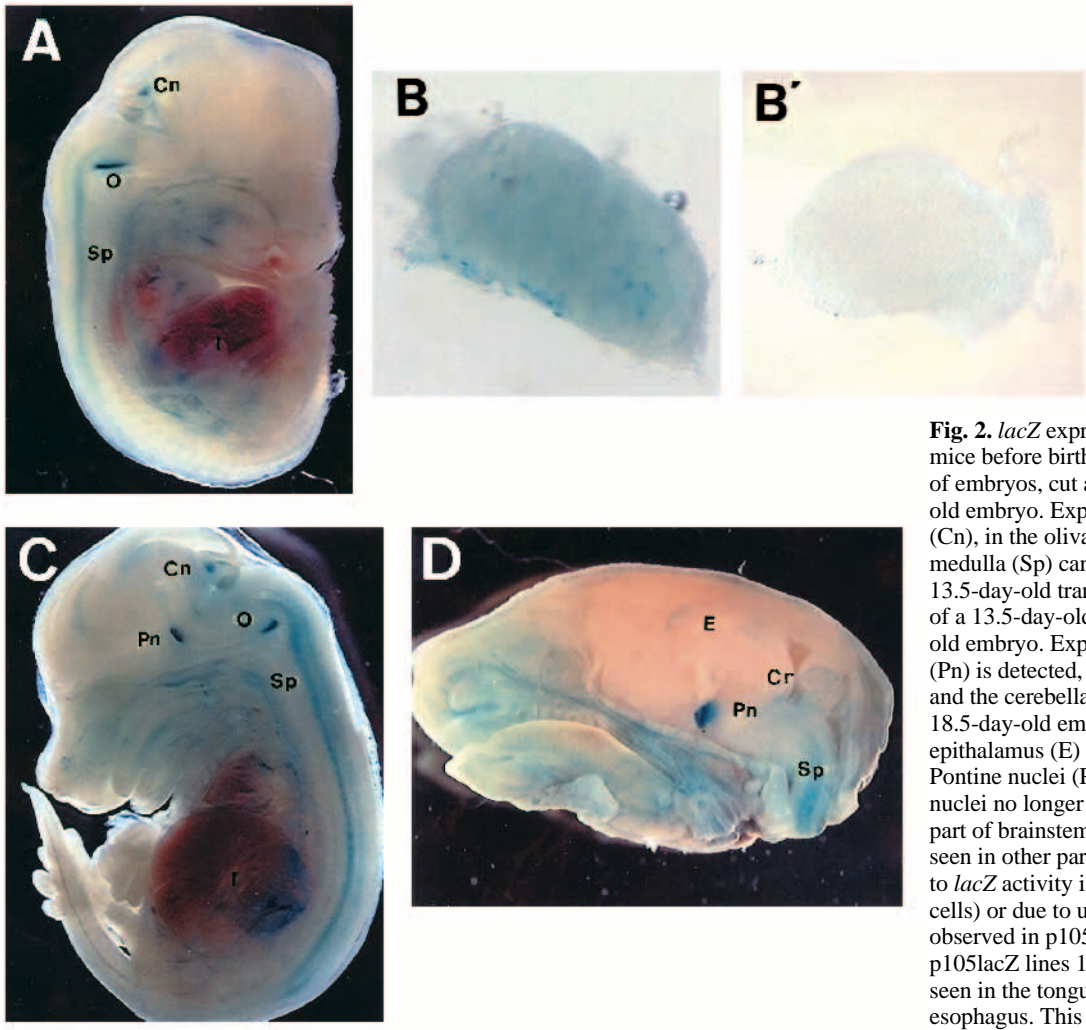


Fig. 2. *lacZ* expression pattern in the 189-4 mice before birth. Whole-mount X-gal staining of embryos, cut along the spine. (A) 12.5-day-old embryo. Expression in the cerebellar nuclei (Cn), in the olivary nuclei (O) and in the spinal medulla (Sp) can be seen. (B) Thymus of a 13.5-day-old transgenic embryo. (B') Thymus of a 13.5-day-old control embryo (C) 14.5-day-old embryo. Expression in the pontine nuclei (Pn) is detected, in addition to the olivary (O) and the cerebellar nuclei (Cn). (D) Head of a 18.5-day-old embryo. Weak expression in the epithalamus (E) starts to be visible at this point. Pontine nuclei (Pn) stain very strongly. Olivary nuclei no longer stain positive in the central part of brainstem (see text). The blue staining seen in other parts of the embryos is either due to *lacZ* activity in the blood vessels (endothelial cells) or due to unspecific staining, equally observed in p105lacZ $-/-$ littermates. In the p105lacZ lines 189-4 (shown here) staining is seen in the tongue and in the lining of the esophagus. This was not seen in any other line.

the fixative, organs were quickly dried, snap-frozen in liquid nitrogen and stored at -80°C . The β -galactosidase reaction was performed on 5 μm and 20 μm frozen sections as described above. Tissue sections were counterstained with Harris hematoxylin.

Morphological identification of the cells that showed *lacZ* activity was done on the 5 μm sections. The precise mapping and quantification of cells expressing the *lacZ* gene was performed on 20 μm sections with the Biocom hardware analysis system (Les Ulis, France) and the Historag software from Biocom. Briefly, this image analysis system allows the different regions of the tissue section to be projected onto a video screen at low magnification. Therefore, the position of the cells is defined relatively to each region, allowing a precise quantification of *lacZ*-positive cells. Counting of cells was done manually under visual control on both video screen and microscope in order to avoid artifacts of automatic thresholding.

Gel mobility shift assay

Nuclear extracts were prepared and gel mobility shift assays were performed as previously described (Ten et al., 1992), using the κB site derived from the enhancer of the immunoglobulin κ light chain gene ($\text{Ig}\kappa$) as a probe. For whole-cell extracts, tissues were homogenized in a minimal volume of buffer containing 20 mM Hepes pH 7.0, 0.15 mM EDTA, 0.15 mM EGTA, 10 mM KCl, 0.15 mM spermidine, 0.2% NP40, 0.4 M NaCl, 1 mM PMSF, 1 mM DTT, 5 $\mu\text{g}/\text{ml}$ leupeptin, 5 $\mu\text{g}/\text{ml}$ aprotinin and incubated for 30 minutes at 4°C with constant agitation. Following centrifugation, the supernatant was recovered and used for gel mobility shift assays using as a probe the κB site located in the promoter of the MHC class I H-2 K^{b} gene. Sera raised against murine p50 (#1263), p65 (#1226), p52 (#1267) and c-rel (#1051) were kind gifts of N. Rice (Frederick, USA). Serum against relB was kindly provided by R. Bravo (Princeton, USA).

RESULTS

Generation of transgenic mice containing a NF- κB -dependent *lacZ* reporter gene

To examine the spatial and temporal distribution of nuclear NF- κB /Rel activity in the whole organism, we generated transgenic lines carrying a κB -dependent NLS-*lacZ* (β -galactosidase) reporter gene. The *Escherichia coli lacZ* gene has been selected as a reporter gene because of the high sensitivity and convenience of the β -galactosidase assay in both whole mounts and histological sections. The nuclear localization sequence (NLS) hooked to the N terminus of the β -galactosidase targets the protein to the nucleus, thus allowing easy discrimination between specific and diffuse non-specific signals.

We used two different promoters containing several κB -binding sites to control the expression of the *lacZ* reporter gene. The first one consisted of a 3.1 kbp fragment of the p105 gene promoter. The p105 gene encodes the $105 \times 10^3 M_r$ precursor of the NF- κB p50 protein. Its promoter contains three κB -binding sites and is regulated by NF- κB /Rel (Ten et al., 1992; Cogswell et al., 1993). The other promoter construct was composed of three direct repeats of the NF- κB -binding site derived from the immunoglobulin kappa light chain ($\text{Ig}\kappa$) enhancer, placed upstream of the conalbumin (cona) minimal promoter.

Prior to microinjection into fertilized mouse eggs, the various *lacZ* constructs were tested by transient transfection experiments using 293T cells (Fig. 1). The 3.1 kb fragment of the p105 promoter (p105*lacZ*) revealed a very low basal activity (lane 1). By contrast, when the cells were treated with

phorbol esters (PMA, lane 3), or tumor necrosis factor alpha (TNF, lane 4), or were cotransfected with an expression vector for NF- κB p65 (lane 2), the *lacZ* activity was stimulated up to 15 times. When the three κB -binding sites in the p105 promoter were mutated (p105mut*lacZ*), the induction was totally abolished, demonstrating that these sites mediate the PMA and TNF response (lanes 5-8). The results obtained with the ($\text{Ig}\kappa$)₃con*lacZ* construct were similar to the ones described for the p105 promoter (lanes 9-12), with a strong induction by PMA (up to 19-fold) and respectively 5-fold and 10-fold with a p65 expression vector or TNF (lanes 10-12). The control construct with the conalbumin minimal promoter alone (con*lacZ*) did not display any significant activity upon induction (lanes 13-16). Thus, both promoters behaved in a similar way, exhibiting a low basal activity and a strong induction capacity when using classical NF- κB /Rel-activating signals. However, the ($\text{Ig}\kappa$)₃con*lacZ* construct seemed slightly more sensitive than p105*lacZ*. Judging by the above results, we postulated that the two constructs would act as good *in vivo* sensors of nuclear NF- κB /Rel activity. We therefore generated transgenic lines with these two constructs and their corresponding negative controls.

Concerning the p105*lacZ* lines, from seven transgenic lines, three expressed the transgene. For the negative control, p105mut*lacZ*, we obtained eight transgenic lines. Some ectopic expression was observed in the central nervous system in two of these eight lines, but was not due to NF- κB specific *lacZ* activity (data not shown). For ($\text{Ig}\kappa$)₃con*lacZ*, from six transgenic lines, two expressed the transgene. Concerning the negative control of ($\text{Ig}\kappa$)₃con*lacZ*, con*lacZ*, from seven transgenic lines, none showed any β -galactosidase activity. Mice positive for expression were used for further mating and establishment of homozygous lines. Generation 2 (G_2) embryos were analyzed for embryonic expression pattern, as well as adults from generation G_1 and G_2 .

Two lines of the p105*lacZ* mice, 189-4 (1 copy of the transgene) and 410-9 (20 copies of the transgene), were identical in terms of expression pattern and intensity. One of the ($\text{Ig}\kappa$)₃con*lacZ* lines, $\kappa\text{c}197$ -6, had an expression pattern similar to that of lines 189-4 and 410-9. The third p105*lacZ* line, 189-2, basically shared the pattern of the other p105*lacZ* lines, but showed a much weaker expression in the cortex of the adult brain (see below). These characteristics were shared with the second ($\text{Ig}\kappa$)₃con*lacZ* line, $\kappa\text{c}252$ -1. The fact that all positive lines exhibit an almost identical expression pattern, although with some variability in the intensity, is taken as evidence that this pattern is the result of specific activation through the κB -binding sites. Generally, the *lacZ* activity observed for the ($\text{Ig}\kappa$)₃con*lacZ* lines was stronger, as expected from the transfection studies. Following we will present a detailed analysis of the features shared by the different p105*lacZ* and ($\text{Ig}\kappa$)₃*lacZ* lines, and use p105*lacZ* line 189-4 as a paradigm.

lacZ expression pattern of the transgenic mice before birth

We analyzed all preimplantation and postimplantation stages for β -galactosidase (*lacZ*) activity. No activity was discovered at any preimplantation stage nor at later stages up to E12.5 (data not shown). The first *lacZ* activity was found on day E12.5 (Fig. 2A), day E12 still being negative. It was localized

in certain nuclei of the rhombencephalon (in the cerebellar and in the olivary nuclei). The spinal medulla also exhibited *lacZ* activity.

E13.5 showed the earliest expression in the thymus (Fig. 2B), which was mainly localized in the thymic medulla and did not change throughout development. Thymic sections of young adult mice support this finding (see below). No changes in the central nervous system were observed on day E13.5.

On day E14.5, expression in the pontine nuclei, also located in the hindbrain, was detected for the first time (Fig. 2C). It should be noted here that the pontine nuclei are stained very strongly in all lines throughout life.

Between day E14.5 and E16, the *lacZ*-positive cells in the olivary nuclei moved from the center of the ventral rhombencephalon to its sides (data not shown; see discussion).

On day E18.5 a weak *lacZ* expression started to appear in the epithalamus, a region localized just above the thalamus and forming part of the limbic system (Fig. 2D). No changes were seen at the above mentioned sites of activity, i.e. thymus, spinal medulla, pontine, cerebellar and olivary nuclei.

Furthermore the two p105*lacZ* lines, 189-4 and 410-9, and the (Ig κ)₃conal*lacZ* line, κ c252-1, exhibited a particularly strong expression in many blood vessels from as early as E12.5 on. The intensity of *lacZ* expression in the various lines did not correlate with the copy numbers of the transgene, but was most likely determined by the sites of integration. The different integration sites may also account for the slight variations seen in the expression patterns of the lines. For example, expression in the fetal liver was only observed in one of the (Ig κ)₃conal*lacZ* lines (data not shown and see Discussion). Yet, overall the p105*lacZ* and the (Ig κ)₃conal*lacZ* lines revealed a similar *lacZ* expression pattern before birth.

***lacZ* expression pattern in the lymphoid organs after birth**

After birth, *lacZ* expression was observed in the two primary lymphoid organs, thymus (Fig. 3A) and bone marrow (data not shown). *lacZ* expression, detected as early as day E13.5 in the medulla of the thymus continued after birth when it was also found in the cortex. However, positive cells in the medulla were seven times more abundant than in the cortex (see Fig. 3A,C). High levels of *lacZ* expression were detected in many histiocytes (Fig. 3B and see below) and in a few endothelial cells (data not shown).

After birth, expression became also detectable in secondary lymphoid organs such as lymph nodes (Fig. 3D), spleen (Fig. 3F) or Peyer's patches of the small intestine (data not shown). Regarding the lymph nodes, the most striking feature was the detection of strong *lacZ* expression in all sinus-lining cells of the capsula at the periphery of the organ (Fig. 3D). Within the lymph node tissue, low levels of *lacZ* activity were detected in a few cells having lymphocyte morphology, and high levels in some endothelial cells and cells that looked like histiocytes (Fig. 3E). In all transgenic lines, expression in the spleen was restricted to few cells (less than 10 per section; Fig. 3F), but this was to be expected since the organ showed no indications of antigenic stimulation (as is visible from the absence of secondary follicles; see Discussion).

Even if an absolute identification of cells is prevented by both tissue freezing and β -galactosidase reaction, the mor-

phology of the nuclei suggests that three cell types may be identified with some confidence: thymocytes or lymphocytes with a small round nucleus (few of them were positive, and they exhibited a weak activity), endothelial cells with a fusiform nucleus lining the lumen of the vessels and histiocytes with a large ovoid nucleus, scattered within the lymphoid tissue. The histiocytes represent the antigen presenting cells (APC) and correspond to monocytes/macrophages and dendritic cells, that are morphologically indistinguishable on our sections. Together with some endothelial cells lining the blood vessels, they showed a very strong β -galactosidase activity. While many sinus-lining cells in the capsula of the lymph nodes were strongly stained, few scattered blue cells were observed within the tissue of the three lymphoid organs analyzed (spleen, thymus and lymph nodes).

***lacZ* expression pattern after birth in the central nervous system**

Just after birth, on day 0 p.n., no changes were seen compared to day E18.5. On day 1 p.n. first expression in the colliculus superior and inferior and in the vestibular nuclei was observed (Fig. 4A). Day 6 p.n. revealed the first, still very light expression in the cortex (Fig. 4B). Day 8 p.n. showed the first expression in the cerebellum (Fig. 4C). It coincides with the time of maturation of the cerebellar complex. The expression observed in the cerebellum was not ubiquitous, being stronger in the posterior than in the anterior lobes.

Hippocampal expression was not observed until 2 weeks after birth (Fig. 4D). Full *lacZ* expression in the cortex did not manifest itself until about 3 weeks after birth (Fig. 4E), with the exception of the p105*lacZ* line 189-2 and the (Ig κ)₃conal*lacZ* line κ c252-1, where expression in the cortex stayed much weaker throughout life. Although the intensity of expression in the cortex varied from line to line, its staining was noted in all lines. Expression in the central thalamus, the region of the substantia nigra and the protectal zone was detected in all lines as well (see Fig. 4E).

When studying whole mounts or cryosections of adult brain tissue, *lacZ* expression could be observed in the outer layers of the cortex, but not in the very first layer (Fig. 5A). In the hippocampus, expression was detected in the CA1, CA3, the habenulus (data not shown) and the dentate gyrus (Fig. 5C,D). We also found expression in the epithalamus (Fig. 5B), in the vestibular and olivary nuclei (Fig. 5G), in the cerebellar nuclei (Fig. 5F), in the granular layer of the cerebellum (Fig. 5E) and in the pontine nuclei (Fig. 5H) in all lines.

In order to determine whether *lacZ*-positive cells were neurons (as suggested on the basis of morphological criteria; not shown), glia or both, we stained cryosections with antibodies directed against β -gal and NF160 neurofilaments which identify neurons (fig 6A). Colocalisation of nuclear *lacZ* and cytoplasmic neurofilaments in the same cell was shown by confocal microscope analysis, demonstrating that *lacZ*-positive cells are essentially neurons. Cryostat sections were also stained with anti- β -gal and anti-GFAP (glial fibrillary acidic protein) antibodies which identify the glial cells: no glial cell stained positive for β -galactosidase activity (Fig 6B). In the cerebellum of all p105*lacZ* lines and the (Ig κ)₃conal*lacZ* line κ c252-1, *lacZ* expression was restricted to the granule neurons.

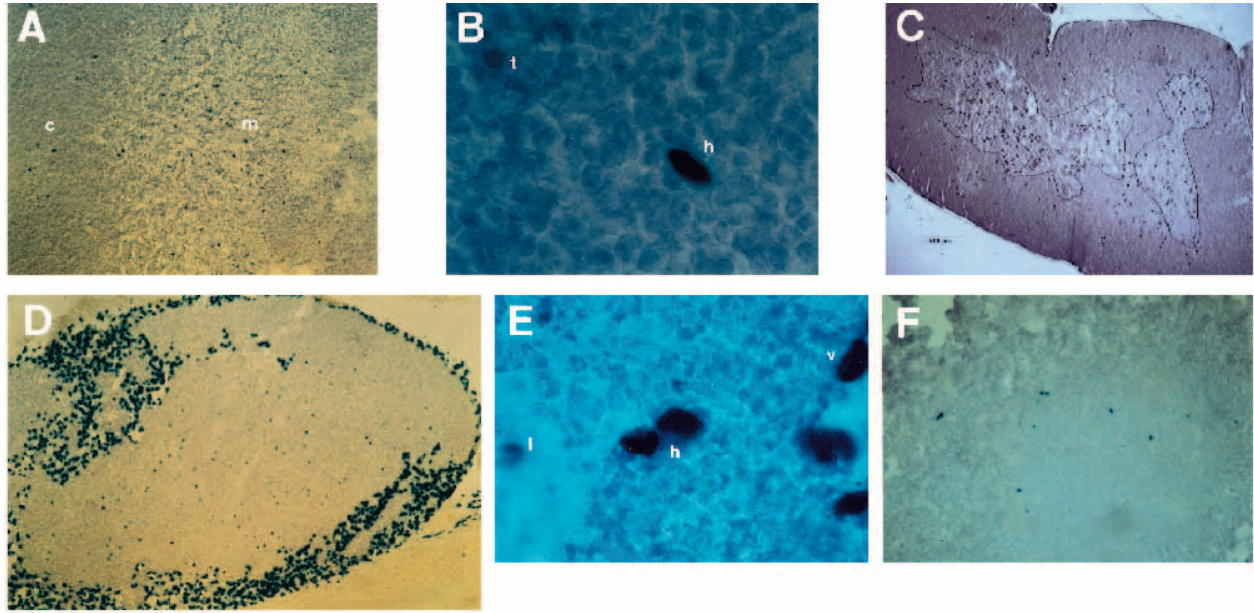


Fig. 3. *lacZ* expression in the lymphoid organs of adult mice. (A) Thymus section (100 \times) with the cortex (c) and the medulla (m). (B) The medulla of the same thymus as in A, 1000 \times magnification. One thymocyte (t) and one histiocyte (h) are indicated. (C) Mapping of κ B-dependent *lacZ* activity on thymus section. The frequency of cells with a high β -galactosidase activity was 1.2×10^{-3} and 8.1×10^{-3} for the cortex and the medulla respectively. (D) Section of a lymph node (100 \times). The *lacZ* activity is detected in all sinus lining cells forming the capsula of the lymph node and in some scattered cells within the lymphoid tissue. (E) The same lymph node as in D, 1000 \times magnification. One lymphocyte (l), one histiocyte (h) and one vessel (v) are indicated. (F) Spleen section with a white pulp islet (160 \times).

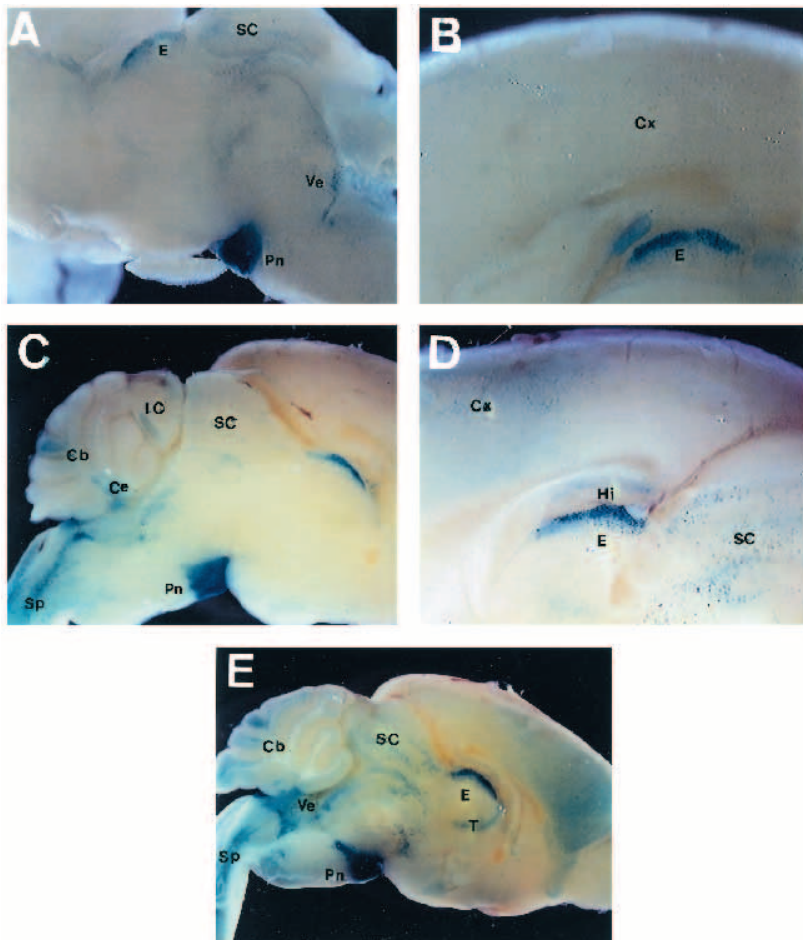


Fig. 4. *lacZ* expression in the brain of the 189-4 mice after birth. Whole-mount X-gal staining of brain, dissected between the two hemispheres. (A) Day 1 p.n.: expression in the epithalamus, the colliculus superior, the vestibular nuclei, the pontine nuclei and cerebellar nuclei can be seen. (B) Day 6 p.n.: a weak expression in the cerebellar cortex starts to be detectable. The epithalamus stains very strongly. (C) Day 8 p.n.: first expression in the cerebellum. Note the irregular staining already seen at this stage. (D) Day 15 p.n.: first expression in the hippocampus. Staining of the cortex, the epithalamus and the colliculus is seen here as well. (E) Day 21 p.n.: adult expression pattern. Note the strong expression in the pontine nuclei and the stronger staining of the posterior lobules of the cerebellum. Cx, cortex; Cb, cerebellum; Ce, cerebellar nuclei; SC, colliculus superior; IC, colliculus inferior; E, epithalamus; Hi, hippocampus; Pn, pontine nuclei; Sp, spinal medulla; T, thalamus; Ve, vestibular nuclei

Correlation between *lacZ* expression and nuclear NF- κ B/Rel-binding activity in the transgenic mice

We examined whether the expression of *lacZ* in our transgenic mice was directly correlated with nuclear NF- κ B/Rel-binding activity, as postulated from our transient in vitro transfection experiments (see Fig. 1). Assuming that free (non I κ B-associated) NF- κ B in whole cell extracts corresponds to nuclear activity, gel mobility shift experiments were carried out with whole cell extracts from several lymphoid organs or regions of the brain incubated with the canonical κ B site located in the promoter of the MHC class I gene H-2 K^b.

Fig. 7, lane 1, shows that two complexes, I and II, could be detected in axillary and iliac resting lymph nodes. To identify the proteins responsible for the formation of these complexes, we used antisera directed against each of the five known mammalian members of the NF- κ B/Rel family. Serum against p50, alone (lane 2) or in combination with anti-p52 serum (lane 3) reduced the intensity of complex I, inhibited the formation of complex II and led to the formation of an intense supershifted complex. The anti-p65 serum slightly decreased the intensity of complex I and induced the formation of a faint supershifted complex (lane 4). Complexes I and II remained unchanged after incubation with anti-c-rel serum (lane 5). Anti-relB serum alone induced the formation of a weak supershifted complex and markedly reduced the intensity of complex I (lane 6). In combination with serum against p65 (lane 7), anti-relB serum almost completely abolished formation of complex I without significantly affecting formation of complex II. The weak remaining complex could be abolished by further addition of anti-c-rel serum (lane 8). From these results, we can conclude that complex I mainly consists of the heterodimeric species p50/p65, p50/relB and p50/c-rel. Complex II corresponds to p50/p50 homodimers. We thus demonstrate that a constitutive nuclear κ B-binding activity exists in resting lymph nodes. This activity is consistent with the β -galactosidase expression detected in this tissue by in situ staining (Fig. 3D). Experiments carried out with other *lacZ*-positive lymphoid organs such as thymus or spleen from healthy mice also revealed a constitutive nuclear κ B-binding activity (data not shown).

We next analyzed extracts from defined regions of the brain, another *lacZ*-positive organ. Fig. 8, lane 1, reveals the presence of two specific complexes, I and II, in extracts from pontine nuclei. Anti-p50 serum abolished both κ B-binding activities and led

to the formation of an intense supershifted complex (lanes 2 and 4), while anti-p65 antiserum eliminated only complex I (lanes 5, 8 and 9). Antisera against p52 (lane 3), c-rel (lane 6) or relB (lane 7) had no effect indicating that p52, c-rel and relB are absent from these complexes. We thus conclude that, in pontine nuclei, complex I consists of p50/p65 heterodimers and

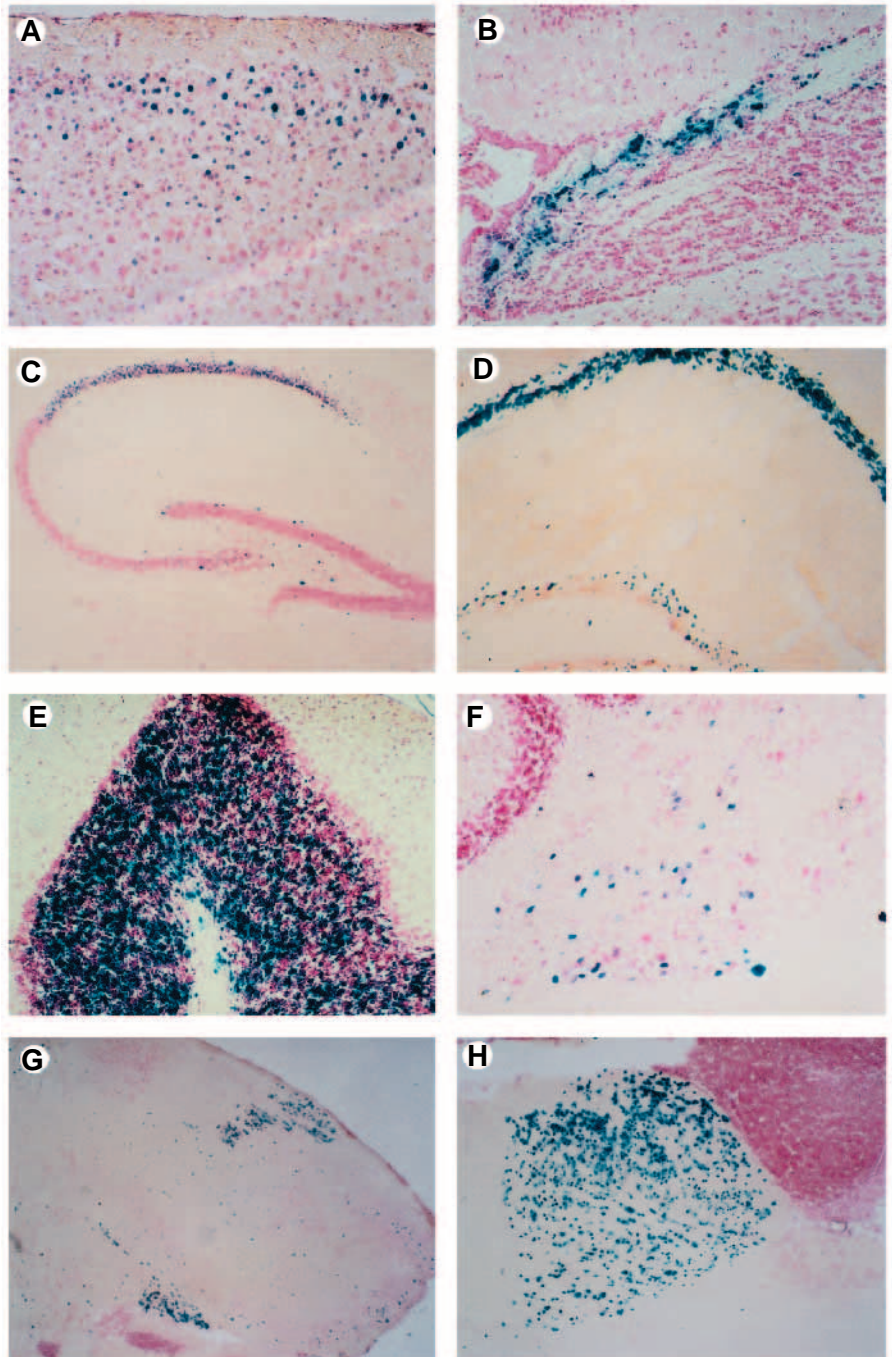


Fig. 5. *lacZ* expression in the adult brain. Parasagittal cryo-sections of adult brains, counterstained with neutral red. (A) Sections of the cortex. Note that the outer layers are not labeled. (B) Staining of the epithalamic region. (C,D) CA1 and CA3 fields of Ammon's Horn and the dentate gyrus. (E) Section of a posterior lobe of the cerebellum. Note the strong staining in the granular layer (granule cells). (F) Section of cerebellum showing the cerebellar nuclei. (G) Section through lateral brainstem: staining of olivary and vestibular nuclei. (H) Section of the region of pontine nuclei.

complex II of p50/p50 homodimers. Similar results were obtained with whole-cell extracts from hippocampus (data not shown). These nuclear κ B-binding activities correlate with the β -galactosidase expression detected in these two regions of the brain by *in situ* staining (Figs 2C, 4C,D, 5C,D,G). On the contrary, when extracts from hypothalamus, which did not exhibit any *lacZ* expression, were used, only one specific κ B-binding complex could be detected (Fig. 8, lane 10). This activity is sensitive to anti-p50 serum (Fig. 8, lane 11) but not to anti-p65 serum (lane 12), indicating that only p50/p50 homodimers are present in this *lacZ*-negative tissue.

Taken together, these data demonstrate that β -galactosidase expression in our transgenic mice is directly correlated with nuclear NF- κ B/Rel activity, such activity being undetectable in negative tissue (Fig. 8, lane 10 and Fig. 9A, lane 1). Moreover, the presence of p50/p50 homodimers alone does not seem to be sufficient for driving κ B-dependent expression *in vivo*. In brain, p50/p65 heterodimers appear to be responsible for such activity.

Inducibility of *lacZ* expression in response to NF- κ B/Rel activation in transgenic mice

We have shown above that, in our transgenic mice, β -galactosidase expression is a marker of constitutive nuclear NF- κ B/Rel activity. We next asked whether this β -galactosidase expression was responsive to classical NF- κ B/Rel-inducing agents. We treated embryonic fibroblasts from p105nslacZ transgenic mice (line 189-4) with LPS, TNF or IL1 β for 1.5 hours to obtain nuclear extracts for use in gel mobility shift experiments, or for 20 hours to reveal β -galactosidase expression by X-Gal staining. Unstimulated fibroblasts did not exhibit any specific nuclear NF- κ B/Rel-binding activity (Fig. 9A, lane 1). As expected, no β -galactosidase expression could be detected either (Fig. 9B). After treatment with IL1 β , LPS or TNF, a specific κ B-binding complex appeared (Fig. 9A, lanes 2-4). This inducible κ B-binding activity was reactive to both anti-p50 and anti-p65 sera indicating that it was made up of p50/p65 heterodimers (lanes 5 and 6). Consistently, β -galactosidase expression became apparent after treatment by any of the three inducing molecules (Fig. 9B), and the percentage of *lacZ*-positive cells roughly correlated with the intensity of the retarded complex (Fig. 9A). These results show that, in our transgenic mice, β -galactosidase activity behaves as a *bona fide* sensor of NF- κ B/Rel-binding activity, whether constitutive or inducible.

DISCUSSION

The aim of our analysis was to study NF- κ B/Rel activity during mouse development and in adult tissues. Using different promoter constructs, which could only be activated if nuclear NF- κ B/Rel complexes were present in the cell, we produced several transgenic mouse lines. The reporter gene consisted of the β -galactosidase gene (*lacZ*). We have shown that detectable *lacZ* expression in our transgenic lines corresponded to nuclear NF- κ B/Rel activity and was absent in cells that did not exhibit such activity. Furthermore, we have clear evidence that tissues such as embryonic fibroblasts, which show no specific NF- κ B/Rel activity prior to stimulation, do so after being stimulated with classical inducers of NF- κ B/Rel.

Moreover, the NF- κ B-dependency of our transgenes was confirmed *in vivo* by the absence of *lacZ* activity in their negative control mice. Therefore these NF- κ B-dependent *lacZ* transgenic mice give us the unique opportunity to analyze the spatiotemporal pattern of NF- κ B activity in a mammal. Since the β -galactosidase detection assay that we use is quite sensitive (it can detect less than 500 copies of β -galactosidase molecules per cell; Vernet et al., 1993), it is unlikely that any additional nuclear NF- κ B activity has been overlooked.

NF- κ B/Rel activity during mouse embryogenesis

Our results showed that NF- κ B/Rel activity appeared for the first time on day E12.5. This observation leads to the conclusion that NF- κ B/Rel activity starts late in mammalian development, a fact that was already evoked by Carrasco et al. (1993, 1994), concerning relB and c-rel expression during mouse development: earliest expression was reported on day E13.5 for the NF- κ B c-rel protein in the thymus and in the fetal liver. However, we did not find *lacZ*-positive cells in the fetal liver, except in the (Ig κ)₃conalacZ line κ C197-6, where it could first be detected around day E13.5 and might correspond to ectopic expression due to the site of integration (data not shown). It must be stressed that constitutive NF- κ B activity in the fetal liver has not been demonstrated previously: expression of c-rel only has only been observed by immunocytochemistry (Carrasco et al., 1994) and no gel mobility shift assay has been performed establishing any constitutive nuclear complexes.

Compared to *Drosophila melanogaster*, where the DORSAL protein starts to be functional very early in development (St. Johnston and Nüsslein-Volhard, 1992), NF- κ B/Rel in mammals seems not to be active until the cells of a particular tissue have matured, which means after completion of developmental processes. In addition NF- κ B/Rel knock-out experiments (Beg et al., 1995; Köntgen et al., 1995; Sha et al., 1995; Weih et al., 1995) support this hypothesis as mentioned in the introduction. Therefore, in mammals, NF- κ B/Rel does not seem to play a role in developing and differentiating a tissue, but rather in maintaining its function, once matured. Yet we cannot exclude the possibility that there might be new undiscovered members of the NF- κ B/Rel family present early in development, which might bind to sites that are slightly different from Ig κ . Experiments are in progress to address these questions.

The results from our transgenic mice indicate that the earliest activity is detected in the central nervous system. On day E12.5 activity was located in the olivary nuclei, in the cerebellar nuclei of the rhombencephalon and in the medulla of the spinal cord. Interestingly, the activity in the olivary nuclei was first observed in the center of the ventral rhombencephalon, migrating towards the lateral parts around day E15. This correlates with the fact that the neurons forming the olivary nuclei and other brainstem nuclei originate in the dorsal alar plate of the rhombencephalon. From here they start to migrate towards the ventral part of the rhombencephalon and later towards its lateral parts, a process known as rhombic migration (Paxinos, 1985). From day E14.5 on, activity in the pontine nuclei could be found. Activity in these three areas remained throughout life. The future hindbrain structures and the cerebellum emerge from the rhombencephalon, which is the first to mature during development.

NF- κ B/Rel activity in the lymphoid organs and other tissues of the adult mouse

NF- κ B/Rel activity was found both in primary and secondary lymphoid organs. It appeared in the medulla of the thymus as early as E13.5, at a time that coincides with maturation of the immune system. In the adult, *lacZ* expression was mostly found in the resident antigen-presenting cells, i.e. dendritic cells and monocytes/macrophages from T cell areas, but less in thymocytes. This finding supports the results on *relB* expression in the thymus (Carrasco et al., 1993), which seems to be restricted to the dendritic antigen presenting cells. In the spleen, we observed very few positive cells, which were not identified as lymphocytes but as endothelial cells lining some vessels and histiocytes. In the lymph nodes, we detected very strong staining in sinus-lining cells of the capsula and some dendritic cells in the medulla. The former are endothelial cells bordering the lumen of lymphatic vessels. This strong NF- κ B/Rel activity is related to the function of these cells, which is to govern and permit the homing of afferent lymphocytes within the lymph node. In this regard, it has been demonstrated that adhesion molecules involved in leukocyte diapedesis, like E-selectin, ICAM1 and VCAM1 are regulated by NF- κ B/Rel (Whelan et al., 1991; Iademarco et al., 1992; Kaszubska et al., 1993; Shu et al., 1993). *RelB* is also known to be expressed in endothelial cells. When we analyzed the NF- κ B/Rel complexes found in this organ, *relB/p50* heterodimers were encountered, confirming that *relB*-containing complexes may account for the major complex responsible for constitutive NF- κ B/Rel activity in the lymphoid tissues (Lernbecher et al., 1993; Weih et al., 1994).

These data are consistent with the results obtained by Lernbecher et al. (1993), who have produced transgenic lines containing the κ B motif from the immunoglobulin kappa chain enhancer as well, but upstream of the β -globin gene. They analyzed the adult mice for constitutive transgene expression by RNase mapping (therefore precluding analysis of individual cells), and found expression in lymphoid tissues, such as thymus and spleen. These organs harbour predominantly immature T and mature B and T cells, respectively. They concluded that constitutive *p50/relB* is the major complex found in these organs. This was confirmed by an independent study (Weih et al., 1994). The analysis by another group of the NF- κ B/Rel complexes present during B cell ontogeny concludes that *relB*-containing complexes are only present in plasmacytoma cells, not normally found in secondary lymphoid organs (Liou et al., 1994).

Interestingly very few positive cells belonging to the lymphocyte type (thymocytes, mature lymphocytes of the lymph nodes or of the spleen) could be detected, and *lacZ* staining was always weak. However, drawing conclusions about the absence of nuclear NF- κ B/Rel activity in lymphocytes from secondary organs is hazardous; indeed examination of both secondary lymphoid organs showed that they are unstimulated, with a complete absence of secondary follicles. This apparent absence of antigenic stimulation is probably due to clean housing conditions. Therefore the vast majority of lymphocytes would be continuously recirculating resting cells. From our results, no conclusion can be drawn about the potential role of NF- κ B proteins during elaboration of an immune response in secondary lymphoid organs. Experiments are in progress to characterize the pattern of *lacZ* expression following antigenic

stimulation. A related question is whether the approach that we used is able to detect a transient stimulation. It has been reported that the half-life of the β -galactosidase protein in neurons is between 24 and 48 hours (Smith et al., 1995). Therefore, if this value holds true for other tissues as well, it indicates that transient bursts of NF- κ B activation induced by short-term stimuli might possibly be detectable.

The *p105lacZ* transgenic lines 189-4 and 410-9 and the (Ig κ)₃conalacZ line κ c252-1 revealed expression in the collecting tubules and in the pelvis of the kidney (data not shown). To our knowledge, there are no studies demonstrating NF- κ B/Rel activity in the kidney. Yet, recent findings indicate that hyperosmotic shock induces NF- κ B/Rel transactivation (G. Courtois and A. Israël, unpublished data). Experiments are in progress to address the possible relevance of this observation.

Adult liver generally did not show any NF- κ B/Rel activity, except in a few cases. It is known that the adult liver does not have constitutive NF- κ B/Rel activity unless hepatectomy is performed (Tewari et al., 1992; Cressman et al., 1994). Therefore removal of the liver and possible damage may account for the *lacZ* activity found in our case.

NF- κ B/Rel activity in the central nervous system

The earliest NF- κ B/Rel activity in our transgenic animals was observed in several nuclei of the brainstem, which are composed of a conglomeration of neurons. Shortly before birth expression in the epithalamus was detected. Major changes were not observed until after birth, when the structures associated with the cortex and the cerebellum start their final maturation process, which is a sequential event that also involves the activation of a particular set of genes (Kuhar et al., 1993). The appearance of NF- κ B/Rel activity in a given part of the brain seems to coincide with the time of its maturation. For instance in our case, the cerebellum does not show any activity before day 8 p.n.. This result is supported by recent data (Guerrini et al., 1995), which demonstrate that NF- κ B/Rel is inducible before day 8 p.n. (days 3-7 p.n.) in the granule neurons of the cerebellum. However, after day 7 p.n., NF- κ B/Rel activity is constitutive and can no longer be induced. The maturation process of the cortex does not end until about 3 weeks after birth and may even continue thereafter. We see first signs of NF- κ B activity from day 6 p.n. on, but the full adult expression pattern is not achieved until around day 21 p.n.

The *lacZ*-positive cells in the brain were essentially neurons, in accordance with recent publications showing that there is constitutive NF- κ B/Rel activity in adult neurons (Bakalkin et al., 1993; Kaltschmidt et al., 1993, 1994; Rattner et al., 1993). This activity mainly consists of *p50/p65* heterodimers. Although these complexes are generally considered as inducible forms of NF- κ B/Rel, in contrast to the constitutive *relB*-containing complexes, they seem to represent a constitutive form of NF- κ B in the brain. However, the long half-life of the β -galactosidase protein in neurons (Smith et al., 1995) does not allow us to rule out the possibility that we might detect local microinductions of NF- κ B. This would be consistent with the fact that a fraction of the inducible forms of NF- κ B has been found in the synapses (Kaltschmidt et al., 1993). In addition to members of the NF- κ B/Rel family, Korner et al. (1989) have identified a factor called BETA, which binds κ B

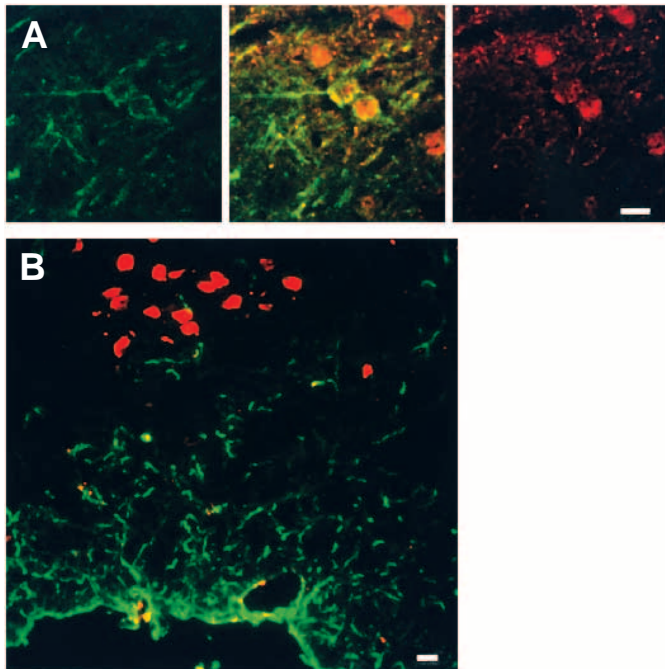


Fig. 6. Identification of the cells expressing the *lacZ* gene in the brain. (A) Colocalisation of β -gal and neurofilaments in neurons from the parietal cortex. Cryostat sections reveal fluorescein-labelled neurofilaments NF160 in the cytoplasm (left panel) and Texas-Red-labelled β -gal in the nucleus (right panel). Optical sections (1.5 μ m) were selected with a confocal microscope to ensure that the localisation was in the same plane. Slight overlaps of the two signals in the nucleus and the cytoplasm are seen in yellow and allow to unambiguously identify *lacZ*-positive cells as neurons (middle panel). (B) Mutually exclusive labelling of GFAP (Glial fibrillary acidic protein)-positive glial cells and *lacZ*-positive cells in the parietal cortex. Cryostat sections show Texas-Red-labelled β -gal in the nucleus and fluorescein-labelled GFAP in the cytoplasm. Bar, 10 μ m.

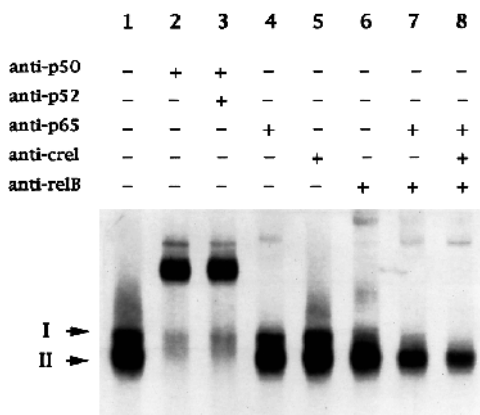


Fig. 7. Constitutive nuclear NF- κ B/Rel-binding activity in resting lymph nodes. Whole-cell extracts prepared from resting lymph nodes dissected out from F₁ B6 \times SJL hybrid mice were incubated with a double-stranded oligonucleotide corresponding to a canonical κ B site located in the promoter of the MHC class I gene H-2 K^b. The addition of specific antisera against the different members of the Rel/NF- κ B family is indicated at the top. Lane 1 corresponds to extracts incubated with preimmune serum. The arrows indicate the different NF- κ B/Rel complexes discussed in the text.

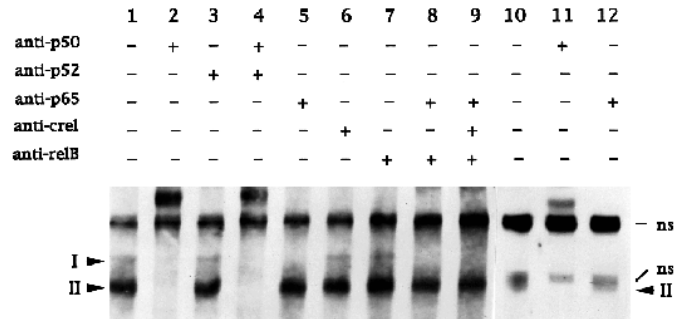


Fig. 8. Constitutive nuclear NF- κ B/Rel-binding activity in the brain. Whole-cell extracts prepared from pontine nuclei, lanes 1 to 9, or from hypothalamus, lanes 10 to 12, dissected out from F₁ B6 \times SJL hybrid mice were incubated with a double-stranded oligonucleotide corresponding to a canonical κ B site located in the promoter of the MHC class I gene H-2 K^b. The addition of specific antisera against the different members of the Rel/NF- κ B family is indicated at the top. Lanes 1 and 10 correspond to extracts incubated with preimmune serum. The arrows point out the different NF- κ B/Rel complexes discussed in the text. ns, non-specific bands. Longer exposure of the film did not reveal any additional complex.

sites in the brain and is a tissue specific and constitutive nuclear protein, although it has not been characterized further.

Nothing is known about the function of the NF- κ B/Rel family in the central nervous system. Data from the targeted disruption of p65, p50 and c-rel did not give any hints of a possible involvement of NF- κ B/Rel proteins in the functioning of the brain. p65 knock-out mice die around day E16 (Beg et al., 1995), too early to allow detection of any defect in the CNS, considering that most of the NF- κ B/Rel activity in the brain is not observed until after birth in our transgenic mice. Since p65 is included in all NF- κ B/Rel complexes in the brain (see above), it seems very likely that it is the major activator in the complex. This may account for the fact that no CNS phenotype has been observed in the p50 or in the c-rel knock-out. On the contrary, NF- κ B p50, c-rel or p65 could be substituted by other, as yet unidentified, members of the family in the brain, or by other transcription factors altogether.

The sites of NF- κ B/Rel activity in the brain are too diverse to assume a possible common function. The only conclusion that we can draw from our results and the results of others is the presence of NF- κ B/Rel activity in mature neurons, and the fact that these proteins do not play a fundamental role in developmental processes of the brain. It is rather unlikely that NF- κ B/Rel activity is induced by one particular neurotransmitter. It rather seems that they are responsible for maintaining basic physiological functions of the neurons. Adhesion molecules may be a candidate target of NF- κ B/Rel regulation in the brain. One may also speculate that NF- κ B/Rel regulates different target genes in the various parts of the brain. Further studies are required to reveal the function of NF- κ B/Rel in the brain and to identify its target genes.

The κ B-dependent *lacZ* transgenic mice described here represent the first available tool allowing a detailed cell by cell analysis of the pattern of NF- κ B activity in various tissues and at different stages of development. In addition, they will be of valuable use for the analysis of the physiological role of the NF- κ B/Rel family in the living animal, as well as for assaying drugs suspected to interfere with the immune or inflammatory

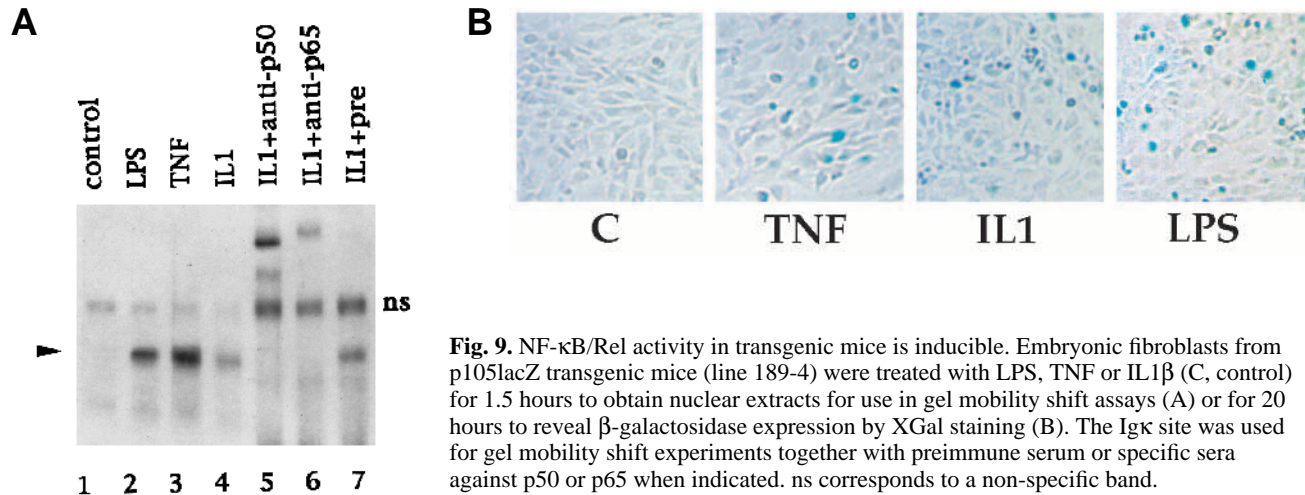


Fig. 9. NF- κ B/Rel activity in transgenic mice is inducible. Embryonic fibroblasts from p105lacZ transgenic mice (line 189-4) were treated with LPS, TNF or IL1 β (C, control) for 1.5 hours to obtain nuclear extracts for use in gel mobility shift assays (A) or for 20 hours to reveal β -galactosidase expression by XGal staining (B). The Ig κ site was used for gel mobility shift experiments together with preimmune serum or specific sera against p50 or p65 when indicated. ns corresponds to a non-specific band.

response. In particular, it will be interesting to analyze the crossing of these mice with knock-out mice for the different members of the family or for transcription factors that have been shown to interact with NF- κ B. Another possibility is to cross our mice with mice overexpressing these various proteins, or with mice transgenic for the Tax protein of HTLV1, which is known to activate NF- κ B in certain tissues. Some of these experiments are in progress.

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